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# The impact of Pickering intervention on the stability of $W_1/O/W_2$ double emulsions of relevance to foods

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## ABSTRACT

Although water-in-oil-in-water ( $W_1/O/W_2$ ) double emulsions have been associated with a spectrum of potential applications in foods, their complex microstructure is significantly unstable. Pickering stabilisation, reputed for superior and longer-term interfacial stabilisation when compared to surfactant-stabilised systems, could provide the opportunity to enhance double emulsion stability. The current work presents a systematic study on the impact of progressively adopting such a Pickering intervention onto one or both interfaces of  $W_1/O/W_2$  emulsions relevant to foods. A range of surfactants/emulsifiers and particles have been used at the  $W_1/O$  or  $O/W_2$  interface of the  $W_1/O/W_2$  microstructure and, where appropriate, cross-compared with the equivalent interfaces of simple emulsions ( $W/O$  and  $O/W$ , respectively). As the aqueous compartments of all investigated systems were not osmotically balanced (at the point of

formulating/forming these), any advantages in terms of double emulsion stability enhancement can be directly attributed to the employed particle stabilisation. It is demonstrated that although partial Pickering intervention can encourage stability (particularly if that is introduced at the inner  $W_1/O$  interface), only complete Pickering stabilisation of the double microstructure can ensure that oil globule size is maintained and internal water phase is retained unaltered over a storage period of one month.

**KEYWORDS:** Pickering stabilisation;  $W_1/O/W_2$  double emulsions; foods; Laplace pressure.

## INTRODUCTION

Double emulsions or multiple emulsions are three-phase assemblies described as ‘emulsions of emulsions’.<sup>(1),(2)</sup> Commonly, their complex microstructure takes the form of a water-in-oil ( $W_1/O$ ) emulsion dispersed within a secondary aqueous phase ( $W_2$ ) in order to produce a final water-in-oil-in-water ( $W_1/O/W_2$ ) double emulsion.<sup>(3),(4)</sup> In the food industry in particular, such double emulsions can be utilised to improve/facilitate the encapsulation of active ingredients such as vitamins and minerals<sup>(5)-(8)</sup> and for ‘unperceived’ fat reduction in emulsions<sup>(9)-(12)</sup>. However, despite their numerous potential food applications, double emulsions have not been reported in industrial applications. The main reason for this is their inherent propensity to rapidly undergo destabilisation.<sup>(13),(14)</sup> In  $W_1/O/W_2$  emulsions, instability primarily stems from the difference in the Laplace pressures between the two aqueous compartments (similarly to Ostwald ripening), which promotes water migration/exchange and can lead to the eventual breakdown of the duplex architecture.<sup>(14),(15)</sup> Aqueous mass transfer can be accelerated by the type/concentration of the surfactants employed to stabilise the two interfaces.<sup>(13),(14)</sup> It has been extensively shown that

incorporation of small molecules (usually salts or sugars) within one or both of the aqueous parts of the double assembly can enhance stability by providing an osmotic opposition to the Laplace differential.<sup>(13),(15)</sup> Alternatively, water exchange can be restricted by ‘structuring’ the oil phase.<sup>(16)</sup>

An approach that may also enhance the integrity of the double emulsion architecture is the introduction of Pickering stabilisation within the system. The adsorption of colloidal (micro- or nano-) solid particles at the oil-water interface can provide (Pickering) emulsions of superior stability.<sup>(17)</sup> A number of studies provide evidence of prolonged double emulsion stability through the use of particle-surfactant combinations. Examples of solid particles used in these approaches include microcrystalline cellulose<sup>(18)</sup>, cellulose nanofibrils,<sup>(19)</sup> colloidal starch,<sup>(20)</sup> kafirin nanoparticles<sup>(21)</sup>, fat crystals<sup>(22)</sup> and montmorillonite clay<sup>(23)</sup>. However, reports on double emulsions solely stabilised by particles are still scarce. Williams *et al.*<sup>(24)</sup> reported on the formation of stable  $W_1/O/W_2$  emulsions using two types of silica decorated with poly(ethylene imine). In another study, vegetable oil-in-silicone oil-in-vegetable oil (V/S/V) emulsions (containing silica particles of different surface silanol content) were stable against coalescence for over a month.<sup>(25)</sup> In their fundamental study, Oza and Frank used colloidal microcrystalline cellulose (CMCC) to stabilise both the internal and external interfaces of  $W_1/O/W_2$  emulsions;<sup>(18)</sup> however, either Span 80 or Span 85 were also added to the oil phase to facilitate the stabilisation of the inner interface directly (by surfactant adsorption) or indirectly (enabling CMCC particle adsorption). More recently and with specific relevance to food applications, Estrada-Fernández *et al.*<sup>(26)</sup> reported on the use of soluble and insoluble whey protein concentrate-gum arabic complexes for the stabilisation of  $O_1/W/O_2$  emulsions which were stable over 76 days of storage.

However, in addition to the relatively limited number of studies in this area, our current understanding on the impact of a Pickering approach on the stability of double emulsions is

somewhat hindered by the diversity in the approaches employed. As a whole, available literature in this area has considered a high number of colloidal particles, with a range of physicochemical characteristics (size, wettability, etc.), for their capacity to provide stability onto osmotically balanced (or not) double emulsions with different architectures and phase volume fractions, formed using an array of emulsification processes operating under various processing conditions. Therefore, drawing firm conclusions from such varied findings is understandably challenging and a number of significant questions still remain. For example, is Pickering stabilisation of only one of the two interfaces of the duplex microstructure sufficient to ensure the integrity of the double architecture is maintained? If that is the case, then which of the two interfaces should be preferentially targeted? Perhaps it is only through the adoption of a complete Pickering strategy that double emulsion stability can be realised? Even then, does the Laplace pressure difference between the two aqueous compartments of the  $W_1/O/W_2$  microstructure still promotes water migration? Finally, how are all of the above influenced by specific formulation (particle/surfactant type and/or concentration, inner and outer droplet size, etc.) and processing (emulsification method, energy input, etc.) features?

The present work aims to elucidate the impact of Pickering intervention on the stability of  $W_1/O/W_2$  double emulsions that are relevant to food applications. Conventional surfactant-stabilised, partially Pickering-stabilised (where particles are only used to stabilise either the  $O/W_2$  or  $W_1/O$  interface) and solely Pickering-stabilised double architectures have been produced via the same emulsification method and under identical processing conditions. In order to (as much as possible) only focus on the impact of Pickering intervention, none of the investigated double systems were (at the point of formulating/forming these) osmotically balanced. A range of surfactants/emulsifiers (sorbitan monolaurate, sodium stearyl lactylate, sodium caseinate and

polyglycerol polyricinoleate) and particles (colloidal microcrystalline cellulose, hydroxypropyl methylcellulose, rutin hydrate and ethylcellulose) were employed at the  $W_1/O$  or  $O/W_2$  interface of the  $W_1/O/W_2$  microstructure and, where appropriate, cross-compared with the equivalent interfaces of simple emulsions ( $W/O$  and  $O/W$ , respectively). The obtained  $W_1/O/W_2$  microstructures were visualised by light and confocal microscopy, while their oil globule droplet sizes and inner water ( $W_1$ ) content were characterised using multi-angle light scattering and differential scanning calorimetry, respectively.

## **MATERIAL AND METHODS**

### **Material**

Distilled water and commercially available sunflower oil were used for the preparation of all emulsions. The interfacial tension between distilled water and the used commercial sunflower oil was monitored throughout this work (at least on a weekly basis); the average equilibrium interfacial tension for this system was  $24.61 \pm 0.89$  mN/m. Particles studied were rutin hydrate (RH), colloidal microcrystalline cellulose (CMCC), ethylcellulose (EC) and (hydroxypropyl)methyl cellulose (HPMC); all from Sigma (UK). Surfactants used were Tween 20 (sorbitan monolaurate) (T20), from Sigma (UK), and Grindsted® SSL P 55 Veg Kosher (sodium stearyl lactylate) (SSL) and Grinsted® PGPR 90 (polyglycerol polyricinoleate) (PGPR), both from Danisco (UK and Switzerland, respectively). Sodium caseinate (NaCas) from bovine milk, rhodamine B and perylene were from Sigma (UK). All materials were used without purification or modification. Percentages of water and oil phases (presented as %wt.), in addition to emulsifying agents and all other emulsion formulation components, were calculated as the weight of the individual constituent per weight of the final emulsion (simple or multiple).

## Methods

### *Preparation of Pickering particle dispersions*

All particles were introduced into and treated within the continuous phase (at 2.5 wt.%) prior to the addition of the dispersed phase to form the emulsions (either O/W or W/O). 80 g of these particle dispersions were prepared and then heated to 45-50°C for 40 minutes, whilst being agitated with a magnetic stirrer to encourage particle dispersion. Following this, particle dispersions were further processed in a high intensity ultrasonic vibracell processor (Sonics & Materials, Inc., CT, USA) operating at 750 W and 20 kHz.

### *Production of simple emulsions*

For both O/W and W/O emulsions, 20 g of dispersed phase were added to 80 g of the particle dispersion prepared earlier; or to 80 g of a surfactant/emulsifier solution of equivalent concentration. The mixture was emulsified using a rotor-stator mixer (RSM) (Silverson L4RT, emulsion screen diameter 19 mm), for 2 minutes at 10000 rpm.

### *Production of double emulsions*

10% W<sub>1</sub>/O primary emulsions were produced by dispersing 15 g of water into a dispersion consisting of 132 g of sunflower oil and 3 g of EC or PGPR. A small amount of rhodamine B dye ( $\leq 0.05$  wt.%), premixed in an aqueous solution, was included in the dispersed phase as a microscopy marker. 20 g of this W<sub>1</sub>/O emulsion was then dispersed in 80 g of a secondary (W<sub>2</sub>) water phase (made up of 77 g water and 3 g of emulsifier). The final W<sub>1</sub>/O/W<sub>2</sub> emulsion was then produced via low shear RSM processing at 3500 rpm for 2 minutes. In the discussion that follows, double emulsion formulations have all been labelled to denote the emulsifier used at each interface;

e.g., a PGPR/T20 emulsion refers to a  $W_1/O/W_2$  microstructure where PGPR and T20 are used to stabilise the internal ( $W_1/O$ ) and outer ( $O/W_2$ ) interfaces, respectively.

#### *Characterisation of dispersions*

Particle sizes were measured via static multi-angle light scattering (LS) using a Mastersizer Hydro 2000 or Zetasizer Nano ZS (both of Malvern Panalytical, UK). All measurements were carried out in triplicate with mean values given to  $\pm 1$  standard deviation.  $\zeta$ -potential analyses were performed on the Zetasizer Nano ZS, equipped with an MPT-2 multipurpose titration unit. Four drops of a 1 wt.% particle aqueous dispersion were diluted into 25 g of distilled water, mildly shaken for 30 s and then immediately measured.  $\zeta$ -potential measurements were carried out in triplicate and values reported are given to  $\pm 1$  standard deviation.

#### *Characterisation of emulsion microstructure*

Simple and double emulsion microstructure characterisation was carried out after the preparation of each system as well as following different periods of cold (4°C) storage. All emulsion droplet sizes were measured via LS using a Mastersizer Hydro 2000 (Malvern Panalytical, UK). In all cases, (simple and double) emulsion samples were added to a dispersant phase (contained within the external dispersion unit of the instrument) corresponding to the continuous phase of the system being tested; i.e. O/W and  $W_1/O/W_2$  emulsions were added to a water phase, while W/O were added to an oil phase. Please note that in both cases this was a pure water or pure oil phase which did not contain surfactant/emulsifier. All measurements were conducted in triplicate with the mean values given to  $\pm 1$  standard deviation. Where double emulsions are concerned, imaging was particularly important as, in terms of light scattering, double emulsions are optically non-uniform due to the presence of the internal  $W_1$  droplets. The scattering resulting from the internal phase droplets has



not been taken into consideration in the measurement of the oil globule size. It is assumed that the  $W_1$  droplets possess a uniform size and the same refractive index as the oil phase.<sup>(27)</sup> Microscopy images of the double microstructures were taken in conjunction with Mastersizer measurements; both approaches revealed oil globule sizes in close agreement. Light microscopy (LM) was employed for the visualisation (Olympus CH2, Japan, with CCD video camera) of both simple and double emulsions. Double emulsion microstructures were also imaged using a Leica TCS SPE (Leica Microsystems, UK) confocal microscope (CM). A drop of emulsion was placed onto a glass microscope slide and a cover slip was placed over it. Separate fluorescent emission spectra were obtained for the oil (perylene-stained) and internal ( $W_1$ ) water (rhodamine B-stained) phases, enabling spatial identification of each phase within the double microstructures.

#### *Differential Scanning Calorimetry (DSC)*

DSC measurements of  $W_1/O$  and their corresponding  $W_1/O/W_2$  emulsion samples were taken after preparation as well as following one month of storage (DSC samples were stored at room temperature), using a DSC 8000 calorimeter (PerkinElmer Inc., USA). All DSC measurements were conducted in triplicate with the mean values given to  $\pm 1$  standard deviation. For a typical DSC analysis, 5.5 mg of each emulsion were weighed directly into a DSC crucible and then, following a similar protocol to that used elsewhere,<sup>(28),(29)</sup> cooled from 20°C to -60°C at a rate of 5°C/min, held at -60°C to equilibrate for 2 minutes and then heated back up to 20°C at the same rate; the reference crucible was left empty. The reported cooling enthalpies ( $\Delta H$ ) are obtained by integrating the area under the relevant peak in the thermogram and above a linear baseline to the curve using the Pyris software of the instrument. The enthalpy changes during cooling of the double (DE) and the primary (PE) emulsions,  $\Delta H''$  (J/g) and  $\Delta H'$  (J/g) respectively, were calculated

by dividing the enthalpy of the specific transition with the mass of the sample in the measurement pan as follows:

$$\Delta H'' = \frac{\Delta H}{m_{DE}} \quad [1]$$

$$\Delta H' = \frac{\Delta H}{m_{PE}} \quad [2]$$

where  $m_{DE}$  and  $m_{PE}$  are the mass of the double and primary emulsion samples, respectively. The mass fraction of water in the primary emulsion ( $\phi'_{W_1} = 0.2$ ) can be used to calculate the enthalpy change associated with the crystallisation of the  $W_1$  droplets alone ( $\Delta H'_{W_1}$ ) from:

$$\Delta H'_{W_1} = \frac{\Delta H'}{\phi'_{W_1}} \quad [3]$$

Finally, the mass fraction of the primary  $W_1$  droplets subsequently entrapped within the  $W_1/O/W_2$  microstructure ( $\phi''_{W_1}$ ) can be estimated according to:<sup>(28),(30)</sup>

$$\phi''_{W_1} = \frac{\Delta H''}{\Delta H'_{W_1}} \quad [4]$$

and compared to the fraction that in theory would correspond to the complete encapsulation of the  $W_1$  phase within the double architecture ( $\bar{\phi}''_{W_1}$ ); in the present study  $\bar{\phi}''_{W_1} = 0.02$ .

### *Statistical analysis*

All data are presented as mean values  $\pm$  one standard deviation (SD). Statistical significance was determined by performing Student's t-test. Results were considered statistically significant at p-values  $\leq 0.05$ .

## RESULTS & DISCUSSION

### Simple Emulsions

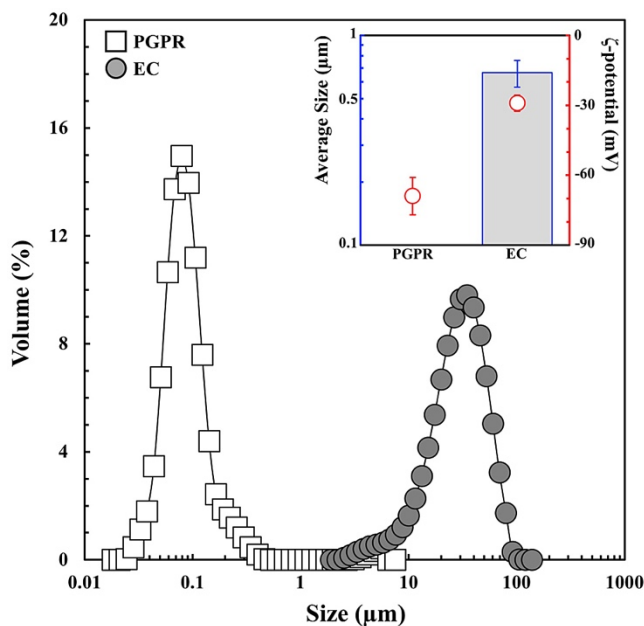
Investigation of the capacity of interfacially active components to provide stability to simple O/W and W/O emulsions serves as an indication of their performance within a  $W_1/O/W_2$  emulsion architecture, where both types of interfaces (primary  $W_1/O$  and secondary  $O/W_2$ ) coexist. This study initially investigated the preparation of both O/W and W/O emulsions using the same emulsification processing conditions later employed for the production of double emulsions. Emulsion stabilisation was previously studied<sup>(31)</sup> for a range of Pickering particles and then compared with that imparted by a number of emulsifiers commonly used to provide stable food emulsions. Informed by these past findings<sup>(31)</sup>, only a selection of colloidal species and emulsifiers of demonstrated W/O and O/W stabilisation capacity were investigated here.

#### *Water-in-oil emulsions*

A key factor that impacts on the overall double emulsion stability is the integrity of the  $W_1/O$  primary interface/emulsion. As it is the first component formed within the to-be-double emulsion assembly, it has to remain stable enough to ensure that both microstructural damage during the secondary processing step and stabiliser migration, between the original  $W_1/O$  interface and the later introduced  $O/W_2$ , are minimised. EC particles were compared to PGPR (a surfactant extensively employed in the stabilisation of  $W/O$ <sup>(32)</sup> and  $W_1/O/W_2$ <sup>(33),(34)</sup> emulsions) in terms of their capacity to produce stable W/O emulsions. The resulting droplet size distributions are shown in Figure 1.

PGPR-stabilised emulsions had significantly smaller droplet sizes than those stabilised using EC particles, irrespective of the dispersed phase (water) content introduced in the system. This in part relates to the much larger size of the EC particles (~664 nm; see inset in Figure 1) compared

to that of PGPR (with a reported hydrodynamic radius of 1 nm)<sup>(35)</sup>, and thus the expected disparity in the interfacial adsorption rates for the two species. What further contributes to this size difference is the change to the oil-water interfacial tension ( $\gamma$ ) induced in each case. EC particles provide only a slight reduction to  $\gamma$  (a value of 21 mN/m has been previously reported)<sup>(31)</sup>, while PGPR results in a much greater interfacial tension decrease; a 2 wt.% PGPR concentration giving an equilibrium value of  $\gamma = \sim 4$  mN/m.<sup>(33)</sup> As the EC-stabilised emulsion droplets are larger than the PGPR-stabilised ones (Figure 1), they are also more prone to sedimentation, which consequently, due to the close proximity of the droplets in the sediment layer, can increase the probability of coalescence and hasten phase separation.<sup>(36)</sup> Nonetheless, both emulsion systems displayed long-term stability, which in the case of the EC-stabilised droplets experiencing sedimentation is hypothesised to be a direct result of Pickering stabilisation.



**Figure 1.** Droplet size distributions of W/O emulsions stabilised by 2 wt.% of PGPR (□) or EC (●), immediately after preparation. Graphs shown are representative of at least three replicate

samples. Inset graph: z-average mean particle diameter for EC (in oil) and  $\zeta$ -potential values for both PGPR and EC. All data points in the inset plots are mean values ( $n = 3$ ); where not visible, error bars are smaller than the used symbols.

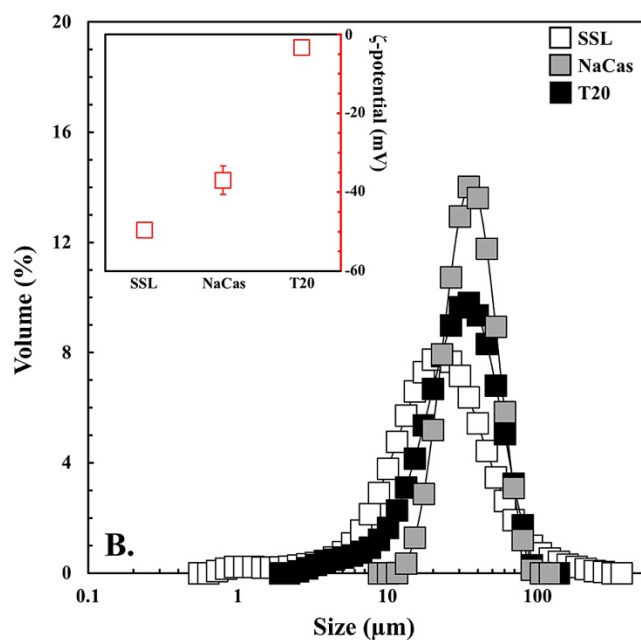
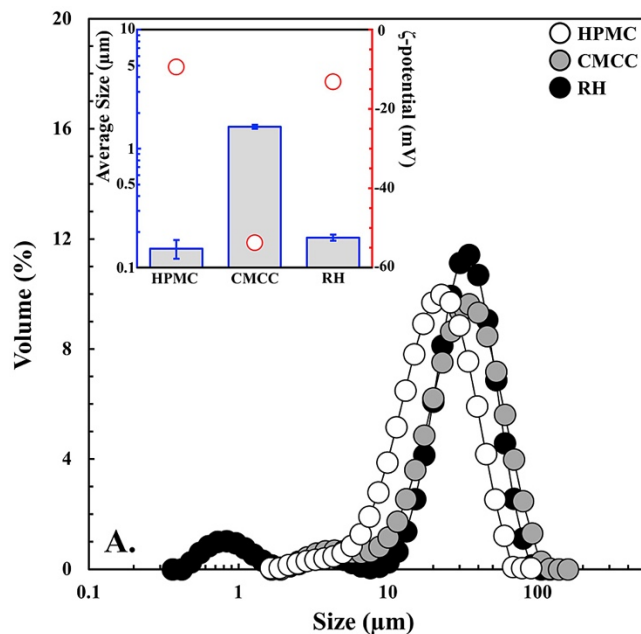
### *Oil-in-water emulsions*

The secondary  $O/W_2$  interface is as critical to the stability of the  $W_1/O/W_2$  emulsion microstructure as the primary one. During the secondary emulsification step, the utilised interfacial species should form a stable layer around the dispersed oil phase (containing the primary  $W_1$  droplets), in order to prevent coalescence events between the oil globules, while at the same time not impacting/disrupting the integrity of the primary  $W_1/O$  interface. Similarly to the work presented earlier for simple W/O emulsions, a range of colloidal species and emulsifiers were investigated here for their potential to provide stable O/W emulsions. CMCC, HPMC and RH were chosen as the colloidal interfacial entities and compared to Tween 20 (T20) and Sodium Caseinate (NaCas). In addition to these, SSL was also tested for its capacity to produce stable O/W emulsions. SSL is an anionic surfactant (typically used as an emulsifier and/or rheology modifier in food products)<sup>(37),(38)</sup> that produces ordered structures (e.g. surfactant bilayers, crystal aggregates) upon dispersion in water.<sup>(39)</sup> It has been previously shown that the fatty acid tails, comprising the surfactant bilayers, crystallise into solid particles upon cooling below the emulsifier's Krafft point.<sup>(37)</sup> As such, despite its surfactant credentials, SSL possesses many of the characteristics usually associated with colloidal particles, when present within an aqueous environment. For this reason SSL has been suggested to stabilise O/W emulsions via a Pickering mechanism.<sup>(40)</sup> Because of its reported dual identity, SSL was used in the present study as an interfacial species acting both as a colloidal particle and an emulsifier; however only for the purpose of reporting the data

associated with its behaviour, SSL is grouped here with the T20 and NaCas emulsifiers. In contrast to the high shear approach used to manufacture the W/O systems, all O/W emulsions were produced using a lower shear process corresponding to the processing environment used for the secondary emulsification step in double emulsion preparation (see methods section). The resulting droplet size distributions, alongside the  $\zeta$ -potential values of all interfacial entities and the particle sizes of only the colloidal species (in water), are presented in Figure 2.

Overall, the obtained data appears to suggest a similar behaviour for all interfacial species (colloidal particles and emulsifiers) in terms of the emulsion droplet sizes produced. The capacity of emulsifiers to lower interfacial tension (an attribute not associated with the interfacial adsorption of colloidal species) coupled with the significant size difference between emulsifiers and particles (and the impact that this has on the rate of interfacial adsorption)<sup>(41)</sup>, would be expected to result in some variation in the size of emulsion droplets stabilised by each species. However, the droplet size distributions of the resulting emulsions are analogous; the average sizes ( $D_{3,2}$ ) of emulsion droplets stabilised by T20 (a highly mobile surfactant that can significantly reduce interfacial tension)<sup>(42)</sup> and those stabilised by CMCC (a colloidal particle of mean dimensions in the order of  $1.5\ \mu\text{m}$ , see inset in Figure 2A, and with no apparent propensity to lower interfacial tension)<sup>(31)</sup>, are  $50\ \mu\text{m}$  and  $54\ \mu\text{m}$ , respectively. The lack of any obvious formulation-related differences between the investigated systems indicates that oil droplet size is controlled by the (low-shear) processing method used rather than the type of the stabilising material employed. Following emulsion production nonetheless, stability under storage conditions is surely expected to be affected by the type of species occupying the formed interfaces. For example,  $\zeta$ -potential can be related to the surface charge at the droplet interface once surfactant or particle adsorption occurs. CMCC, SSL and NaCas had the highest  $\zeta$ -potential (Figure 2) and it would be expected that these

species would form interfacial layers with higher electrostatic repulsion amongst droplets, rendering them more stable against droplet coalescence and emulsion failure during storage. A similar case can be made in terms of steric stabilisation, which is undoubtedly expected to be more prevalent in the case of Pickering stabilised interfaces.



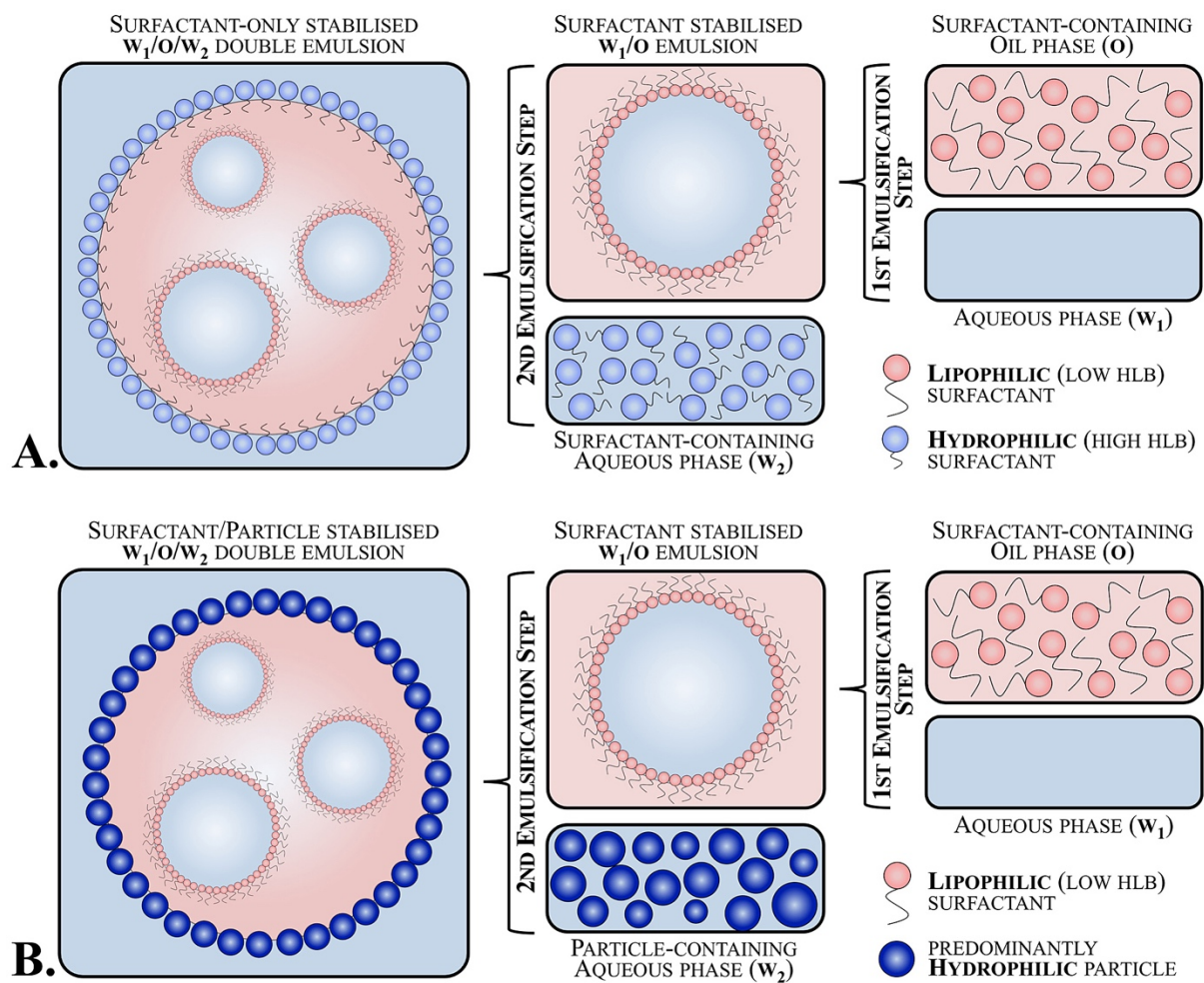
**Figure 2.** Droplet size distributions of O/W emulsions stabilised by 2 wt.% of: **A.** colloidal particles (HPMC, ○; CMCC; ●; RH, ●) or **B.** conventional emulsifiers (SSL, □; NaCas, ■; T20, ■), immediately after preparation. Graphs shown are representative of at least three replicate samples. Also given are the z-average mean particle diameter and  $\zeta$ -potential values for all colloidal particles (inset graph in **A**) and  $\zeta$ -potential values for all emulsifiers (inset graph in **B**). All data points in the inset plots are mean values ( $n = 3$ ); where not visible, error bars are smaller than the used symbols.

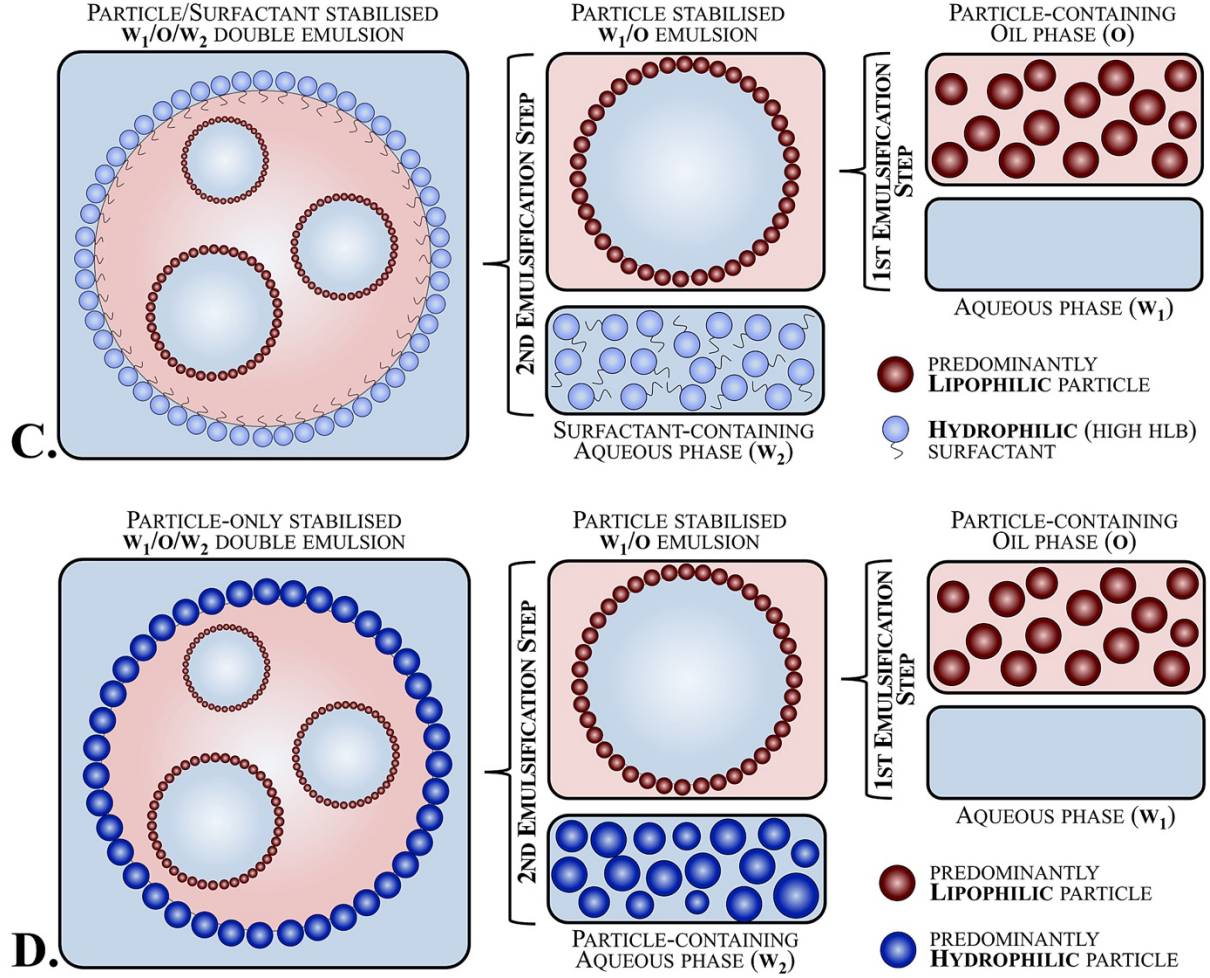
### Double emulsions

Limiting the mobility of interfacial species could offer advantages to the stability of the double emulsion microstructure and thus replacing interfacial stabilisation emanating by surfactants with one arising from the adsorption of colloidal particles (Pickering stabilisation) could prove beneficial.  $W_1/O/W_2$  emulsions were initially prepared using surfactants to stabilise both interfaces. These systems were then used as the basis for comparison against duplex systems where either of the two interfaces was stabilised using Pickering particles or double emulsions where Pickering stabilisation is implemented at both interfaces. As such, four classes of double emulsion microstructures have been investigated (schematically depicted in Figure 3); a conventional surfactant-stabilised double emulsion microstructure (Figure 3A), two partially Pickering-stabilised double assembly, where colloidal particles are used to stabilise either the external  $O/W_2$  (Figure 3B) or internal  $W_1/O$  (Figure 3C) interface, and finally an entirely Pickering-stabilised duplex architecture (Figure 3D). In an attempt to focus on the impact of Pickering stabilisation, all double emulsions were produced via the same processing method and their aqueous phases were not osmotically balanced. It should also be stressed that the



microstructures illustrated in Figure 3 only serve to schematically exemplify the different approaches employed here to study the impact of Pickering intervention on the stability of  $W_1/O/W_2$  double emulsions. As such, the schematics shown here are not in any way meant to portray any physical phenomena (such as surfactant migration between the internal  $W_1/O$  and external  $O/W_2$  double emulsion interfaces) associated with these duplex architectures.



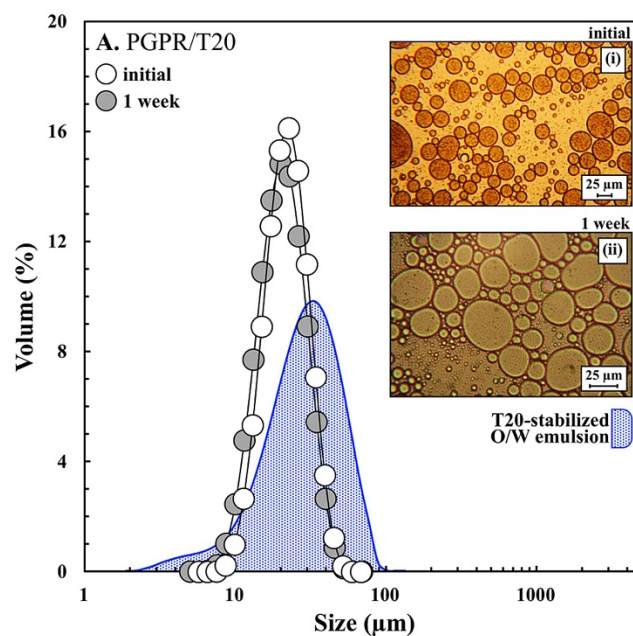


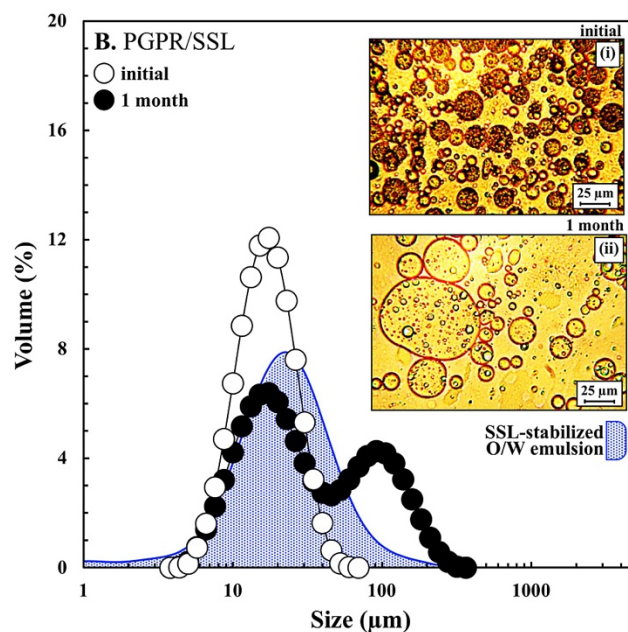
**Figure 3.** Schematic diagram depicting the four different types of double emulsion microstructures of no Pickering stabilisation (A), partial Pickering stabilisation (B and C) and complete Pickering stabilisation (D).

#### *Surfactant-only stabilised $W_1/O/W_2$ double emulsions*

The oil globule size distribution data for the  $W_1/O/W_2$  emulsions stabilised solely by surfactants (PGPR/T20 and PGPR/SSL) are given in Figure 4. Considering only the droplet size data for the simple W/O and O/W emulsions stabilised by PGPR (Figure 1) and T20 and SSL (Figure 2B) respectively, it is expected that, at least in principle, both surfactant-only stabilised

double emulsion systems can be indeed formed (Figure 4A); i.e. the PGPR-stabilised aqueous droplets (in the W/O emulsion) are small enough to be incorporated within the larger T20 or SSL stabilised oil ones (in the O/W emulsion). Therefore unsurprisingly, micrographs taken immediately following processing of these systems confirmed the existence of a double microstructure (Figure 4, insets (i)). In fact, the size distributions for the PGPR/T20 and PGPR/SSL double emulsion oil globules are relatively close to those obtained for simple O/W emulsions stabilised by T20 or SSL (Figure 2B; also included in Figure 4 for direct comparison). Despite this, both PGPR/T20 and PGPR/SSL double emulsions exhibited a high level of instability.





**Figure 4.** Droplet size distributions of  $W_1/O/W_2$  emulsions stabilised by: **A.** PGPR/T20, and **B.** PGPR/SSL; immediately after preparation (○) and following one week (●) and one month (●) of cold storage. The droplet size distribution of a simple O/W emulsion stabilised by either surfactant is additionally shown. All droplet size distributions shown are representative of at least three replicate samples. Inset graphs: light microscopy images of the  $W_1/O/W_2$  emulsions at different time intervals.

In the case of PGPR/T20 double emulsions, the oil globule size distribution following 7 days of storage remained practically unchanged (Figure 4A); also confirmed by the optical microscopy (insets in Figure 4A). Nonetheless, micrographs of these systems revealed a substantial loss of internal  $W_1$  droplets and thus the almost complete collapse of the double microstructure (Figure 4A, inset (ii)). The loss of internal aqueous matter in  $W_1/O/W_2$  emulsions has been extensively reported in literature<sup>(13),(43),(44)</sup> as one of the main destabilisation phenomena leading to the collapse of these systems. This is more prominent in duplex assemblies where the two aqueous

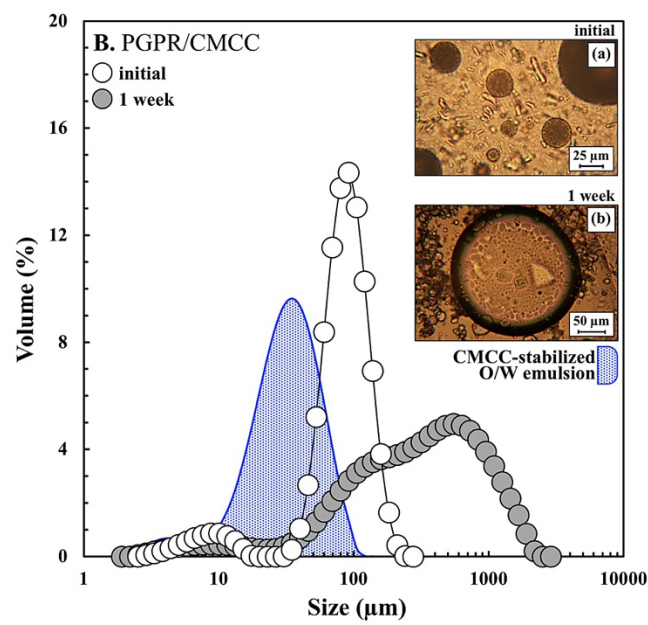
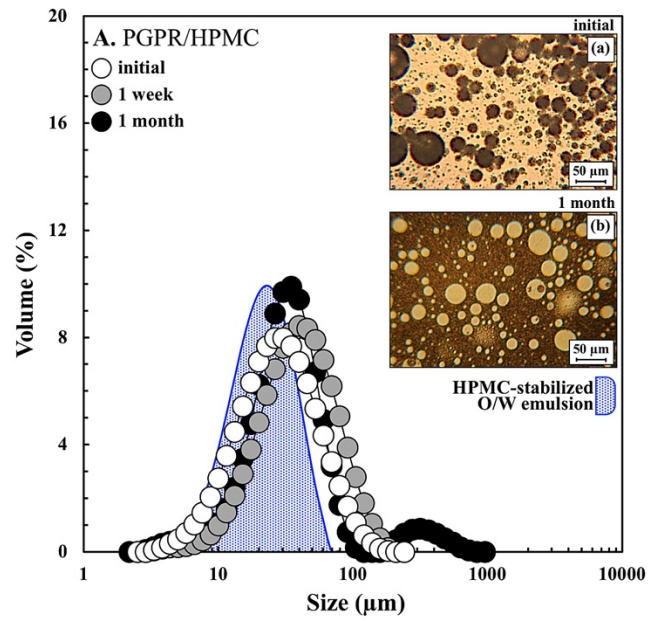
compartments are not osmotically balanced so as to oppose the propensity for destabilisation driven as a result of the inherent Laplace differential that exists between these phases.<sup>(13),(15)</sup> Dragosavac *et al.*<sup>(45)</sup> reported that double emulsions, stabilised by the PGPR/T20 surfactant combination used here, retained an overall similar oil globule size during storage (2 weeks), but were progressively devoid of inner water droplets. Since double emulsions in the same study<sup>(45)</sup> were formed using membrane emulsification (a low shear process delivering droplets one-at-the-time and without comminution), the contribution of the (higher shear in comparison) process used in the present work on the observed loss of  $W_1$  droplets and eventual double emulsion destabilisation can only be deemed to be minimal. As such, the destabilisation process exhibited by the surfactant-only stabilised PGPR/T20 double emulsion system is expected to be principally driven by formulation-related or -assisted mechanisms. As no coalescence of  $W_1$  droplets was observed internally within the oil globules (although relatively challenging to clearly observe, this is also supported by the stability of the simple W/O emulsions stabilised by PGPR),  $W_1$  loss is expected to occur by water transfer across the oil phase and/or by rupture of the oil film and complete expulsion of the inner water droplets into the external aqueous phase.<sup>(13)</sup> Both mechanisms have been shown to be mediated by the presence of surfactants. Water transfer is facilitated by micelle formation and/or spontaneous W/O emulsification in the oil phase, which have been previously shown to take place in the presence of PGPR.<sup>(33)</sup> Similarly, oil film rupture and expulsion of the  $W_1$  droplets as a whole has been suggested to come as a result of surfactant exchange across the two interfaces in the double emulsion system and a subsequent change to interfacial curvatures, leading to the collapse of the microstructure down to a simple O/W architecture.<sup>(13),(22),(43)</sup>

For the PGPR/SSL double emulsions, instability is also proposed to be driven by formulation-specific mechanisms. Although internal  $W_1$  droplets are still present after a month and therefore a double emulsion microstructure (to an extent) still persists, these appear to have grown in size (Figure 4B, insets (i) and (ii)). What is even more pronounced is the change to the oil globule size distribution, which following one month of storage has become bimodal (Figure 4). It is not clear whether this is a result of coalescence events taking place between the oil globules of the PGPR/SSL double emulsions, although this would be in contrast to the stability exhibited by simple O/W emulsions in the presence of SSL.<sup>(40)</sup> As such, coalescence has to be linked to the existence of the PGPR-stabilised  $W_1$  internal droplets. As previously discussed, SSL has been reported to possess a Pickering functionality.<sup>(40)</sup> However, SSL's capacity to form colloidal particles due to its low equilibrium solubility in water, has been reported to diminish in the presence of surfactant micelles (Tween 80).<sup>(46)</sup> Assuming that SSL's performance as an O/W emulsion stabiliser is (at least partially) linked to its colloidal identity, it could be expected that this is affected by the presence of PGPR micelles (similarly to the action of Tween 80 micelles) in the PGPR/SSL double emulsions, resulting in O/ $W_2$  interfaces that have reduced stability and are more prone to coalescence. The observed behaviour could also be related to the swelling of the  $W_1$  aqueous droplets (i.e. increase of the internal aqueous content).<sup>(47)</sup> Although swelling of the internal  $W_1$  droplets in double emulsions is very commonly observed, this is ascribed to an osmotic imbalance (e.g. driven by the presence of KCl or NaCl only within the  $W_1$  droplets) promoting water transport from the  $W_2$  to the  $W_1$  phase;<sup>(13),(15),(47)</sup> thus in the opposite direction to migration induced only due to Laplace pressure differences between the two aqueous compartments (as is the case in the present study).

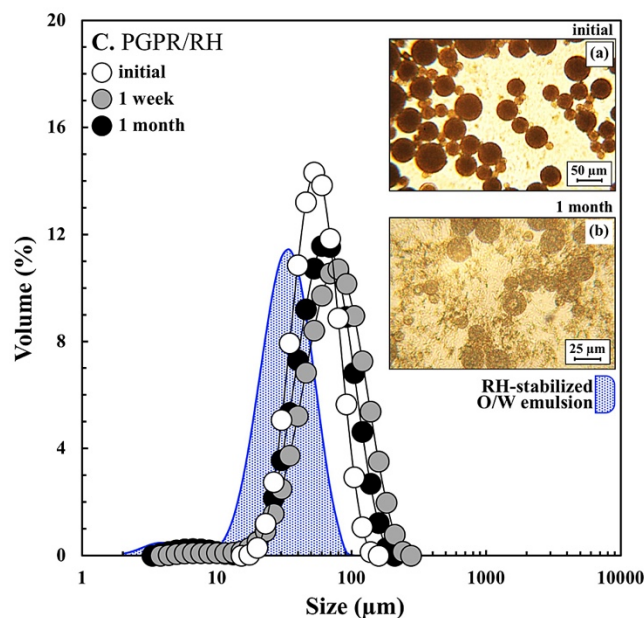
### *Surfactant/Particle stabilised $W_1/O/W_2$ double emulsions*

Partially Pickering-stabilised double emulsions were first produced using a surfactant at the primary interface (PGPR) and colloidal particles (HPMC, CMCC or RH) at the secondary one. The droplet size distributions of the oil globules in the formed PGPR/HPMC, PGPR/CMCC and PGPR/RH double emulsions as well as light micrographs of the produced microstructures are presented in Figure 5. Microscopy images from all systems immediately after preparation confirmed the existence of a double microstructure (Figure 5, insets (a)). In all cases oil globule size distributions are somewhat larger than the size of the oil droplets formed (under the same processing conditions) in simple O/W emulsions stabilised by the same colloidal species (Figure 2A; also included in Figure 5 for direct comparison).









**Figure 5.** Droplet size distributions of  $W_1/O/W_2$  emulsions stabilised by: **A.** PGPR/HPMC, **B.** PGPR/CMCC, and **C.** PGPR/RH; immediately after preparation ( $\circ$ ) and following one week ( $\bullet$ ) and one month of cold storage ( $\bullet$ ). In all cases the droplet size distribution of a simple O/W emulsion stabilised by either of the colloidal species is additionally shown. All droplet size distributions shown are representative of at least three replicate samples. Inset graphs: light microscopy images of the  $W_1/O/W_2$  emulsions at different time intervals.

Despite the successful formation of double emulsions, the three systems exhibited a varying performance in terms of stability. It is worth noting that all colloidal particles studied here have been previously shown to successfully stabilise O/W emulsions for at least two weeks.<sup>(31)</sup> The PGPR/HPMC system largely remained stable in terms of oil globule size (Figure 5A), with the exception of a small peak developing between 100-1000  $\mu\text{m}$  after one month. However, this is overshadowed by the clear and progressive loss of internal  $W_1$  droplets, revealed by microscopy to be all but complete after one month of storage (Figure 5A, inset (b)). The collapse of the

PGPR/HPMC double microstructure resembles that observed for the PGPR/T20 one (Figure 4A) and similarly to the latter surfactant-only system, water migration in these partially Pickering-stabilised double emulsions is promoted by the Laplace pressure difference between  $W_1$  and  $W_2$  and facilitated by PGPR.<sup>(33)</sup>

The PGPR/CMCC system exhibited clear signs of coalescence leading to phase separation after only one week of storage (Figure 5B). Even though both the PGPR/HPMC and PGPR/CMCC systems could not maintain their initially formed double microstructure, there seems to be a difference in the mechanism by which failure progresses and obviously its extent. Although in both cases changes to the double microstructure appear to be related to the presence of PGPR, in the case of the PGPR/HPMC systems interactions between the surfactant and the colloidal particles at least yield a stable  $O/W_2$  interface; albeit eventually within a simple  $O/W$  emulsion assembly. It is proposed that this is due to a synergistic stabilisation mechanism; stable  $O/W$  emulsions have been previously reported in systems co-stabilised by PGPR and HPMC (for surfactant concentrations below 0.1 wt.%), while the same study also shows that  $O/W$  emulsions formed in the presence of higher PGPR concentrations or PGPR alone were unstable.<sup>(48)</sup> Such synergism does not appear to take place in the PGPR/CMCC double emulsions and these systems (in contrast to the PGPR/HPMC ones) do not transform to transiently stable  $O/W$  emulsions but instead phase separate. As such it is believed that PGPR facilitates the exclusion of the previously adsorbed CMCC particles from the external  $O/W_2$  interface and failure of the emulsion microstructure. At this stage it is unclear why the synergism observed between PGPR and HPMC is not observed with CMCC. It is perhaps related to the much lower  $\zeta$ -potential exhibited by CMCC (-54 mV) compared to HPMC (-9 mV), suggesting that it could be the prevalence of repulsive electrostatic interactions between the former and PGPR (-68 mV; Figure 1) that prohibit their interfacial co-

existence. Alternatively (or in addition) the large difference in the sizes of the HPMC and CMCC particles (140 nm and 1.5  $\mu\text{m}$ , respectively; Figure 2A) might also be relevant in this discussion.

Interestingly enough, the PGPR/RH double emulsions displayed the highest level of stability amongst the partially Pickering-stabilised systems investigated here. Not only the oil globule size distribution of these systems remains relatively unchanged during the one-month storage period, but internal  $W_1$  droplets were still visible over the same timeframe (Figure 5C). However, microscopy revealed that although  $W_1$  droplets are present, their population is reduced; note the increase in the optical transparency of the oil globules after storage (Figure 5C, inset (b)). Thus, there is evidence to suggest that water transport due to Laplace pressure differences does take place in the PGPR/RH double emulsions as well. Nonetheless, this does not result (at least not within the present experimental timescales) in collapse into an O/W emulsion (as for PGPR/HPMC systems) or to complete phase separation (as for PGPR/CMCC systems). RH and HPMC are very similar in terms of their particle sizes and  $\zeta$ -potential values (179 nm and 140 nm, and -13 mV and -9 mV, respectively; Figure 2A) and both have the capacity to provide stable O/W emulsions with comparable droplet sizes ( $D_{3,2}$ ) of 47  $\mu\text{m}$  and 36  $\mu\text{m}$ , respectively (Figure 2A; also in Figure 5 for direct comparison). The initial sizes ( $D_{3,2}$ ) of the oil globules in the PGPR/RH and PGPR/HPMC double emulsions are also very close; 71  $\mu\text{m}$  and 67  $\mu\text{m}$ , respectively (Figure 5). Despite these obvious parallels, RH particles possess a strong hydrophilic character; significantly more so than the HPMC (or even the CMCC) particles.<sup>(31)</sup> It could be that this variation in the wettability of the particles and therefore the resulting differences in their positioning at the O/ $W_2$  interface of the double microstructure, impacts on the rate of water transfer between the  $W_1$  and  $W_2$  phases.

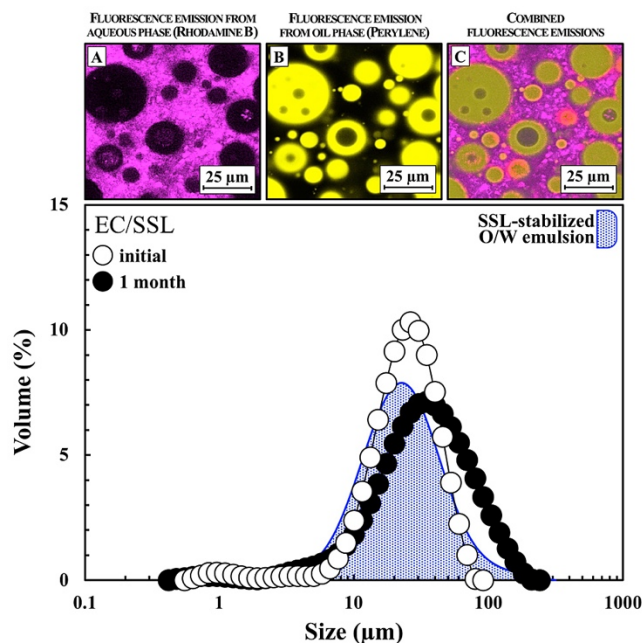
### *Particle/Surfactant stabilised $W_1/O/W_2$ double emulsions*

The second type of partially Pickering-stabilised double emulsions were formed using colloidal species to stabilise the primary interface (EC) and surfactants (T20, SSL) or protein (NaCas) to stabilise the secondary interface. The main difference here, compared to the partially Pickering-stabilised double systems discussed earlier, is the size of the primary emulsion droplets. While PGPR-stabilised  $W_1/O$  emulsions have very small droplet sizes ( $D_{3,2}$  of 200 nm), EC-stabilised  $W_1$  droplets are much larger ( $D_{3,2}$  of 49  $\mu\text{m}$ ) (Figure 1). The entrapment of the EC-stabilised  $W_1/O$  primary emulsions within a duplex microstructure is therefore expected to be more challenging but not physically impossible. Nonetheless, encapsulation efficiency (the fraction of primary droplets retained within a duplex microstructure) in this case is expected to be much lower than that achieved when emulsifying the smaller PGPR-stabilised  $W_1$  droplets; as only the smaller size population of EC-stabilised  $W_1$  droplets can be successfully entrapped.

Initially, partially Pickering-stabilised double emulsions were formed in the presence of EC particles at the primary interface and either T20 or NaCas at the secondary interface. Although both types of interfacial species were earlier shown to provide stable  $W/O$  and  $O/W$  emulsions (for EC (Figure 1) and T20 or NaCas (Figure 2A), respectively), their combination was not effective. Both the EC/T20 and EC/NaCas formulations did not provide interfacial stabilisation, with double emulsions in either case phase separating within hours. Instability in these partially Pickering-stabilised systems is even greater than in the case of surfactant-only (PGPR/T20) stabilised  $W_1/O/W_2$  emulsions (Figure 4A). However, it is also clear that destabilisation here is certainly promoted by the large size of the EC-stabilised  $W_1$  droplets, which the T20-stabilised  $O/W_2$  interface was unable to contain. Even the use of NaCas failed to limit the onset and rapid progression of double emulsion disruption. An additional mechanism can also contribute to the

breakdown of double microstructures stabilised by NaCas. It has been reported that the presence of sodium caseinate in the continuous phase ( $W_2$ ) of a double emulsion can create a significant osmotic pressure imbalance between the two aqueous compartments due to the release of  $\text{Na}^+$  ions (an increased osmotic pressure in  $W_2$ ).<sup>(49)</sup>

Conversely, the EC/SSL stabilised double emulsions were found to be relatively stable; confocal micrographs of these systems show the presence of a double microstructure following 1 month of storage at room temperature (Figure 6). Upon formation, oil globule sizes were analogous to the oil droplet sizes of the SSL-stabilised O/W emulsions. After the one-month storage period however the size of the EC/SSL stabilised double emulsions was found to increase (Figure 6). These systems suffer from the same strain placed on the external interface as the EC/T20 and EC/NaCas stabilised double emulsions; i.e. the attempt to enclose primary (EC-stabilised) emulsion droplets of large sizes within an oil enclosure of comparable (albeit slightly larger) dimensions. Although, as initially anticipated, this results in only a small fraction of  $W_1$  droplets being successfully placed within the oil phase (see confocal micrographs in Figure 6), SSL positioning at the external ( $\text{O}/W_2$ ) interface appears to significantly delay destabilisation phenomena; compared to what T20 or NaCas external stabilisation was shown to afford. It is not entirely clear whether this is in any way associated with SSL's capacity to act in a Pickering fashion. Nevertheless, assuming that double emulsion destabilisation in this case is primarily driven (or at least promoted) by the mobility of the species placed at each interface, it can be hypothesised that the difference in the adsorption/desorption kinetics that is expected to exist between T20 and the colloidal entities formed by SSL, should be a determining factor in the rate of microstructural deterioration.



**Figure 6.** Droplet size distributions of  $W_1/O/W_2$  emulsions stabilised by EC/SSL; immediately after preparation (○) and following one month of cold storage (●). The droplet size distribution of a simple O/W emulsion stabilised by SSL is additionally shown. All droplet size distributions shown are representative of at least three replicate samples. Inset graphs: confocal micrographs of the EC/SSL  $W_1/O/W_2$  emulsion following one month of cold storage. **A.** Fluorescence emission (purple) from rhodamine B, **B.** fluorescence emission (yellow) from perylene, and **C.** combined fluorescence emissions.

#### *Particle-only stabilised $W_1/O/W_2$ double emulsions*

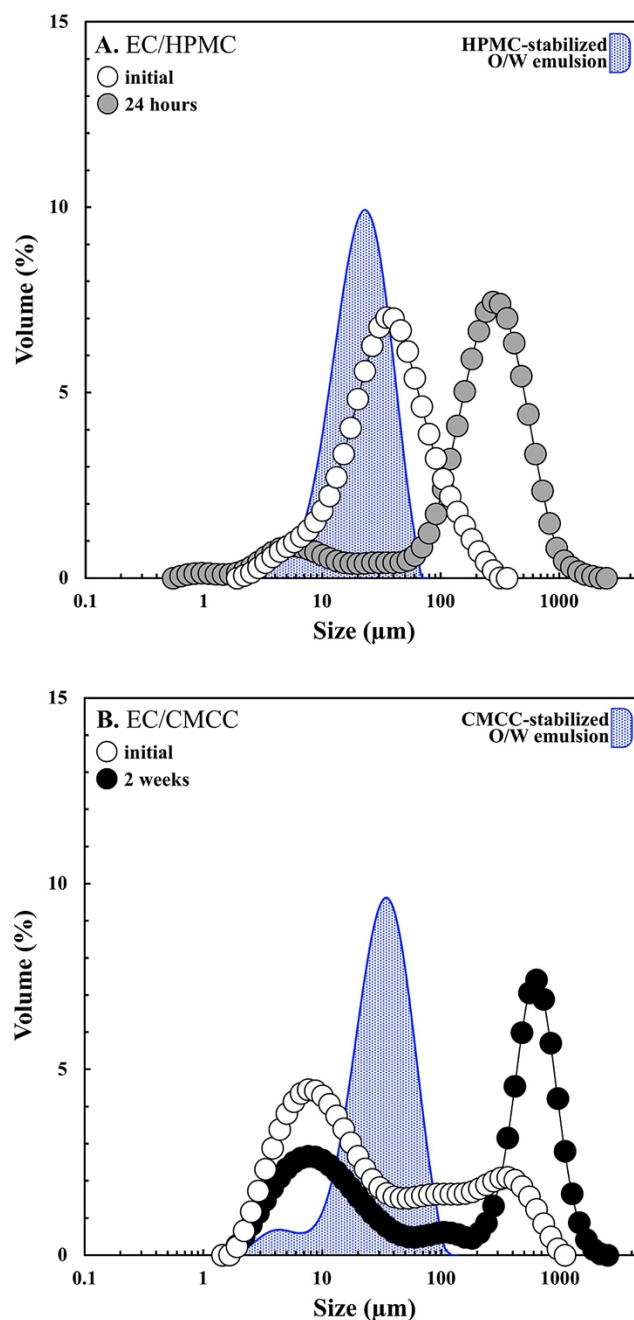
Finally, a series of  $W_1/O/W_2$  emulsions solely stabilised by Pickering particles were produced. EC was used to stabilise the primary interface and HPMC, CMCC or RH colloidal species were utilised for the stabilisation of the secondary interface. Similarly to the particle/surfactant stabilised  $W_1/O/W_2$  double emulsions (EC/T20, EC/SSL and EC/NaCas), the

entrapment of the EC-stabilised  $W_1/O$  primary emulsions within the EC/HPMC, EC/CMCC and EC/RH duplex microstructures was expected to be challenging.

Double emulsions, where CMCC or HPMC were used to stabilise the secondary interface, were not stable (Figure 7). The instability displayed by these systems shows clear parallels to the deterioration exhibited by the PGPR/CMCC and PGPR/HPMC partially Pickering-stabilised double emulsions, and microscopy confirmed that both the EC/HPMC and EC/CMCC systems reverted to O/W emulsion microstructures soon after formation. This may be due to the similarities in their cellulosic physicochemical properties and hence adsorption/competition for the same interface leading to the formation of a simple emulsion rather than a double emulsion; HPMC and EC particles are both predominantly hydrophobic and CMCC has no distinct preference for either the aqueous or lipid phase.<sup>(31)</sup> It is also worth noting that even the simple O/W microstructures, resulting from the collapse of the EC/HPMC and EC/CMCC  $W_1/O/W_2$  emulsions, were not stable against coalescence (Figure 7).

However, external stabilisation by RH particles resulted in the formation of ( $W_1/O/W_2$ ) structures with oil globule sizes that remained unchanged for over 1 month; confocal microscopy also confirmed the presence of double emulsion structures over the same storage period (Figure 8). The superior stability of the EC/RH duplex systems may be due to RH particles being largely hydrophilic, whereas EC particles are predominantly hydrophobic. The difference in the wettability of these two colloidal species is more significant in comparison to that between EC and HPMC or CMCC. As a consequence the EC and RH particles are more likely to remain primarily associated with their original  $W_1/O$  and  $O/W_2$  interfaces (respectively) and not migrate to the opposing interface during the second emulsification step, thus encouraging the stability of the

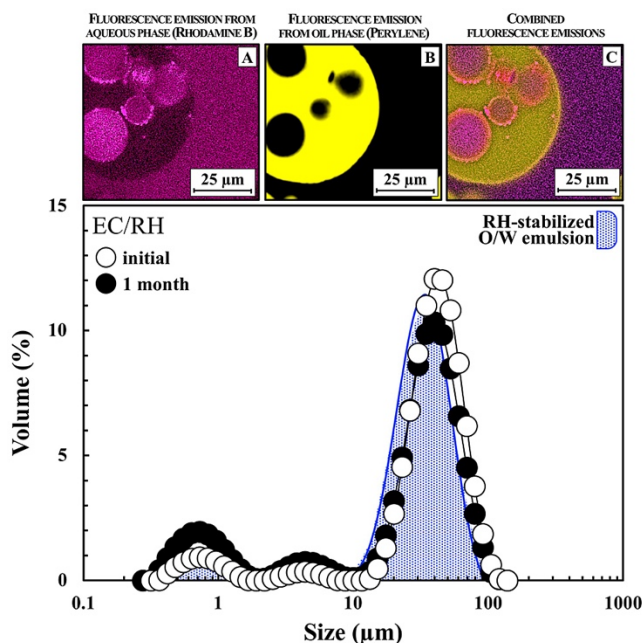
formed double emulsion despite the inherent destabilisation strain imposed by the Laplace differential between their aqueous sections.



**Figure 7.** Droplet size distributions of  $W_1/O/W_2$  emulsions stabilised by: **A.** EC/HPMC, and **B.** EC/CMCC; immediately after preparation (○) and following 24 hours (●) and two weeks of cold



storage (●). In all cases the droplet size distribution of a simple O/W emulsion stabilised by either colloidal species is additionally shown. All droplet size distributions shown are representative of at least three replicate samples.



**Figure 8.** Droplet size distributions of  $W_1/O/W_2$  emulsions stabilised by EC/RH; immediately after preparation (○) and following one month of cold storage (●). The droplet size distribution of a simple O/W emulsion stabilised by RH is additionally shown. All droplet size distributions shown are representative of at least three replicate samples. Inset graphs: confocal micrographs of the EC/RH  $W_1/O/W_2$  emulsion following one month of cold storage. **A.** Fluorescence emission (purple) from rhodamine B, **B.** fluorescence emission (yellow) from perylene, and **C.** combined fluorescence emissions.

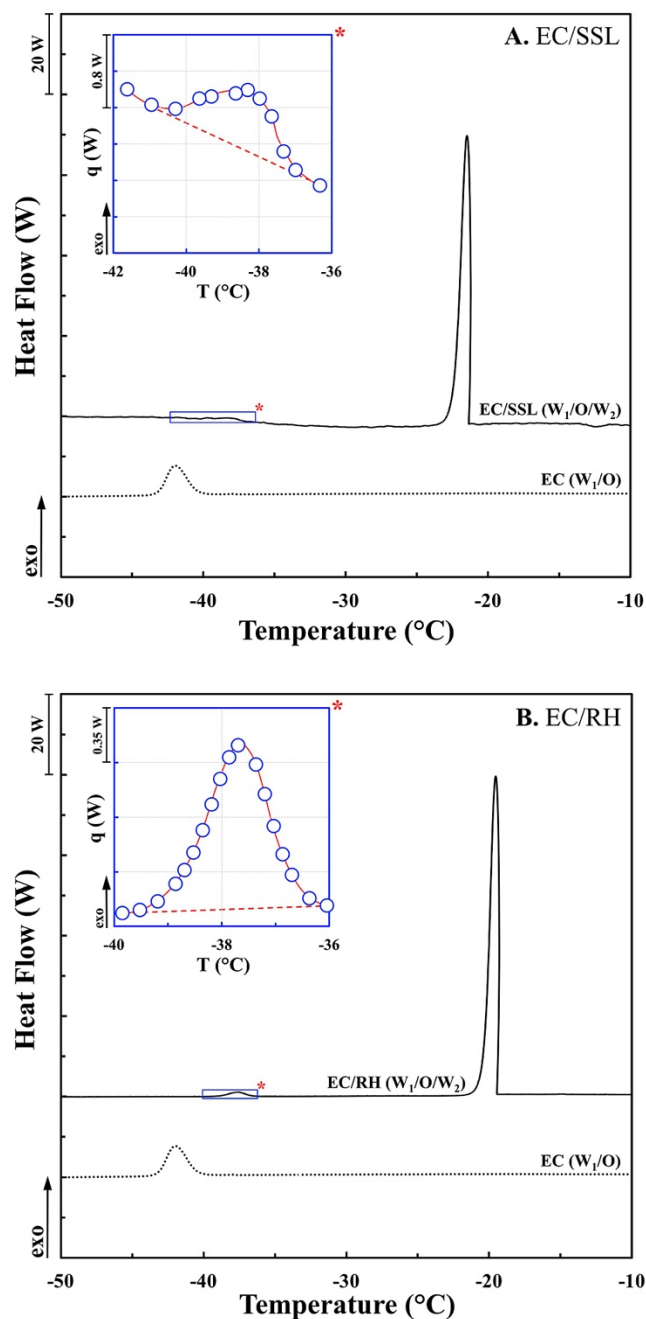
## Calorimetric analysis of the impact of Pickering intervention on double emulsion microstructure

Previous literature reports on the use of differential scanning calorimetry (DSC) analysis for the characterisation of the inner water phase ( $W_1$ ) in double emulsions.<sup>(28)-(30)</sup> The temperature ( $T''_c$ ) during the crystallisation of the internal water phase of the double architecture has been shown to relate to the size of the  $W_1$  droplets.<sup>(28),(29)</sup> In addition, both  $T''_c$  and the enthalpy change ( $\Delta H''$ ; Eq. [1]) can be affected by other parameters such as the presence of additional soluble content (e.g. salts) and emulsifiers/surfactants, as well as by the cooling rate applied during the DSC measurement(s).<sup>(28),(29)</sup> The same influences apply to the temperature and enthalpy values associated with the crystallization of water droplets in the simple ( $W_1/O$ ) primary emulsion;  $T'_c$  and  $\Delta H'$  (Eq. [2]), respectively. By expressing  $\Delta H'$  with respect to the water mass in the primary emulsion ( $\Delta H'_{W_1}$ ; eq. [3]) and comparing this to  $\Delta H''$ , the mass fraction of entrapped  $W_1$  droplets ( $\phi''_{W_1}$ ) within the double emulsion can be estimated (eq. [4]).<sup>(28),(29)</sup> Because of the influences highlighted above, relating  $\Delta H''$  to the crystallization enthalpy of pure water is not appropriate.<sup>(29)</sup> What is more, when the primary emulsion formulation is changed, separate  $\Delta H'_{W_1}$  values should be obtained and then compared to the  $\Delta H''$  enthalpies of their corresponding duplex systems.<sup>(50)</sup>

In the present study, only double microstructures that previously demonstrated the best stability within their category (as set out in Figure 3) were chosen for DSC analysis. Thus, data derived from the DSC curves of double emulsions of no- (PGPR/SSL), partial- (PGPR/RH and EC/SSL) or complete-Pickering stabilisation (EC/RH), were studied to determine how particle stabilisation influences the encapsulation of the inner  $W_1$  phase within the duplex architecture and its fate during storage. The DSC curves obtained for all double microstructures followed the typical pattern previously reported for such systems;<sup>(29)</sup> i.e. an initial peak around  $-20^\circ\text{C}$  (phase transition

of the  $W_2$  aqueous phase; heterogeneous freezing) followed by a second one at  $-40^\circ\text{C}$  (crystallisation of the  $W_1$  aqueous droplets; homogeneous nucleation). A representative example of a DSC curve demonstrating this pattern can be seen in Figure 9. Both initial and storage  $T''_c$  and  $\Delta H''$  ( $\text{J/g}_{\text{DE}}$ ) for these  $W_1/\text{O}/W_2$  emulsions, as well as the  $T'_c$  and  $\Delta H'$  ( $\text{J/g}_{\text{PE}}$ ) values initially measured for their respective  $W_1/\text{O}$  components, are given in Table 1. Also presented are the enthalpy change data for the crystallisation of the  $W_1$  droplets in the  $W_1/\text{O}$  emulsions expressed with reference to the mass content of water alone ( $\Delta H'_{W_1}$ ;  $\text{J/g}_{W_1}$ ) and the estimated mass fractions of entrapped  $W_1$  droplets ( $\phi''_{W_1}$ ), calculated using Eqs. [3] and [4] respectively.

$W_1/\text{O}/W_2$  emulsions utilising PGPR (at the primary  $W_1/\text{O}$  interface) experienced crystallisation of the inner water droplets at a slightly lower  $T''_c$  and had marginally larger  $\Delta H''$  values than the systems where EC was used (Table 1). Overall, this could suggest that double microstructures containing PGPR-stabilised primary emulsions (in comparison to the EC ones) have a larger  $W_1$  content that is potentially also present as smaller droplets.<sup>(29)</sup> Although this is in accordance to the droplet size measurements of the simple  $W/\text{O}$  emulsions stabilised by the two (PGPR or EC) species (Figure 1), it should be noted that only  $T''_c$  and  $\Delta H''$  differences between PGPR/RH and EC/SSL or EC/RH were found to be statistically significant ( $p < 0.05$ ). The  $\phi''_{W_1}$  data (i.e. the fraction of  $W_1$  droplets from the  $W_1/\text{O}$  emulsion that are ultimately retained within the  $W_1/\text{O}/W_2$  microstructure) also suggest that a larger proportion of the PGPR-stabilised  $W_1$  aqueous droplets (compared to the EC-stabilised ones) are captured within the double architecture (Table 1); in this case the  $\phi''_{W_1}$  differences between both systems utilising PGPR and those containing EC were statistically significant ( $p < 0.05$ ).



**Figure 9.** DSC profiles of  $W_1/O/W_2$  emulsions stabilised using EC at the primary interface and either SSL (A) or RH (B) at the secondary interface; for each system, a close-up of the heat flow profile corresponding to the crystallisation of the internal  $W_1$  droplets (section of the main DSC profile within the blue border) is shown as inset. The DSC profile of the EC-stabilised  $W_1/O$  primary emulsion used to produce both double systems is also shown.

**Table 1.**  $T'_c$ ,  $\Delta H'$  (eq. [2]),  $\Delta H'_{w_1}$  (eq. [3]),  $T''_c$ ,  $\Delta H''$  (eq. [1]) and  $\phi''_{w_1}$  (eq. [4]) values for a range of primary  $W_1/O$  and corresponding  $W_1/O/W_2$  emulsions, immediately after formation and following one month of storage at room temperature. PE and DE denote primary and double emulsion, respectively. All measurements were performed in triplicate and data is given as mean values ( $\pm 1$  standard deviation).

$W_1$ in $W_1/O$ ( $\phi'_{w_1}=0.2$ )						
INITIAL (24h)						
	$T'_c$ (°C)	$\Delta H'$ (J/g <sub>PE</sub> )	$\Delta H'_{w_1}$ (J/g <sub>w<sub>1</sub></sub> )			
PGPR	-43.1 (± 1.9)	15.7 (± 3.6)	78.5 (± 18.0)			
EC	-42.0 (± 0.1)	41.0 (± 4.6)	205.0 (± 23.0)			
$W_1$ in $W_1/O/W_2$ ( $\bar{\phi}''_{w_1}=0.02$ )						
	INITIAL (24h)		1 MONTH			
	$T''_c$ (°C)	$\Delta H''$ (J/g <sub>DE</sub> )	$\phi''_{w_1}$ (g <sub>w<sub>1</sub></sub> /g <sub>DE</sub> )	$T''_c$ (°C)	$\Delta H''$ (J/g <sub>DE</sub> )	$\phi''_{w_1}$ (g <sub>w<sub>1</sub></sub> /g <sub>DE</sub> )
PGPR/SSL	-40.1 (± 2.4)	3.8 (± 1.7)	0.048 (±0.024)	—	—	—
PGPR/RH	-41.9 (± 0.8)	5.8 (± 1.8)	0.074 (±0.029)	—	—	—
EC/SSL	-39.3 (± 1.5)	2.2 (± 1.7)	0.011 (±0.008)	-38.0 (± 2.3)	2.6 (± 1.6)	0.013 (±0.008)
EC/RH	-38.4 (± 1.0)	3.4 (± 1.5)	0.017 (±0.008)	-39.6 (± 0.1)	1.7 (± 1.0)	0.008 (±0.005)

With regards to the role of the emulsifier at the secondary ( $O/W_2$ ) interface,  $\phi''_{w_1}$  values appear to suggest that double emulsions where the oil globules are stabilised by RH possess a higher  $W_1$  content, but only if the latter is stabilised by PGPR ( $p < 0.05$ ). Thus, in terms of statistical validity, a larger internal  $W_1$  content (in the form of PGPR-stabilised water droplets) can be enclosed within a RH-stabilised secondary interface than within an SSL-stabilised one. This is

probably because, although both RH and SSL (under processing conditions that are equivalent to those used for double emulsion preparation) give simple O/W emulsions of comparable average droplet sizes (in both cases  $\sim 50\ \mu\text{m}$ ), the SSL-stabilised systems have a much larger population of smaller oil droplets ( $<10\ \mu\text{m}$ ) than those stabilised by RH (Figure 2). In addition, particle-stabilised interfaces have been reported to provide a more robust layer around emulsion droplets,<sup>(51)</sup> thus it could be hypothesised that RH-stabilised oil globules would be less susceptible to loss of  $W_1$  content, during the secondary emulsification step in double emulsion formation, than SSL-stabilised systems. In the case of EC-stabilised water droplets, the aqueous fractions entrapped within double microstructures stabilised externally by either SSL or RH are not statistically different ( $p>0.05$ ). Here,  $\phi''_{W_1}$  values are probably comparable since enclosure within these duplex assemblies is primarily dictated by the much larger dimensions of the EC-stabilised  $W_1$  droplets.

In terms of the stability of the  $W_1$  inclusions within the formed  $W_1/O/W_2$  microstructures, DSC analysis revealed a distinction between double assemblies where the primary emulsion was stabilised by Pickering particles and those where a surfactant (PGPR) was employed for the same purpose (Table 1). More specifically, DSC data suggests that following one month of storage both PGPR/SSL and PGPR/RH double emulsions were devoid of  $W_1$  droplets, while the EC/SSL and EC/RH (Figure 9A and 9B, respectively) still retained some level of internal aqueous content. As previously discussed, droplet size (LS) and confocal microscopy (CM) analyses for the PGPR/SSL (Figure 4B) and PGPR/RH (Figure 5C) double emulsions did reveal evidence of destabilisation and  $W_1$  loss; albeit not the complete lack of internal aqueous content suggested by DSC. This could be a result of the different temperature conditions used for storing samples for LS/CM ( $4^\circ\text{C}$ ) and DSC (room temperature;  $\sim 20^\circ\text{C}$ ) analyses. Therefore, the higher storage temperature for the DSC samples could have contributed to the acceleration of destabilisation phenomena, the progress of

which was slowed down, and thus their effect dampened, in LS or CM samples. Nonetheless, it is clear that  $W_1$  retention within double microstructures containing surfactant-stabilised primary emulsions is problematic. Droplet size data and microscopy images for the EC/SSL (Figure 7) and EC/RH (Figure 8) double emulsions also appears to corroborate the DSC data obtained for these systems. Both systems give  $\phi''_{W_1}$  values that remain practically stable (no statistical difference;  $p>0.05$ ) even after one month of storage. However, given the fact that all other particle/surfactant double emulsions were highly unstable and that SSL has been previously reported<sup>(40)</sup> to possess a level of Pickering functionality, it is uncertain whether this type of partial Pickering intervention would in general prove sufficient in providing long-term stability to  $W_1/O/W_2$  microstructures.

It is worth noting at this point that the estimated  $\phi''_{W_1}$  for double microstructures containing PGPR-stabilised primary emulsions is greater than  $\bar{\phi}''_{W_1}$  (0.02); i.e. the fraction that in theory would correspond to the complete encapsulation of the  $W_1$  phase within the double architecture. This could suggest that there is migration of water from the outer ( $W_2$ ) to the inner ( $W_1$ ) aqueous phase. The systems studied here however have not been subjected to osmotic balancing and the unavoidable Laplace pressure differential within the double microstructure would in theory induce the transfer of aqueous matter in the opposite direction; from  $W_1$  to  $W_2$ . In addition to this, water migration to the internal aqueous phase would have been associated with the swelling of the  $W_1$  droplets and the enlargement of the oil globules containing these. Given the extent of aqueous uptake suggested by the  $\phi''_{W_1}$  data (Table 1), any such increase in the microstructural dimensions of the double emulsions would have certainly been identified during droplet size measurements and even observed in light microscopy analysis; however, neither of these was detected experimentally. A more reasonable hypothesis is to suggest that the observed discrepancy between the  $\phi''_{W_1}$  and  $\bar{\phi}''_{W_1}$  values is as a result of experimental inaccuracies associated with the DSC

measurement itself. It has been suggested that although such errors can occur either during the measurement itself (accuracy of the equipment) or during sampling, these are not expected to contribute to significant inaccuracies, which were estimated to be less than 1%.<sup>(29)</sup> Although this might be indeed true for double emulsions that have been osmotically balanced (as is the case for the systems studied in (29)), DSC measurements of osmotically unbalanced double microstructures suffer from an additional source of experimental inaccuracy. As a typical DSC measurement progresses, a vapor pressure gradient will begin to develop between the outer (frozen) and inner (undercooled) aqueous phases, which inevitably will cause some water migration.<sup>(30)</sup> The addition of solutes in the inner  $W_1$  phase can greatly minimise (or even suppress) the occurrence of water transfer during the DSC measurement.<sup>(30)</sup> The high standard deviation in the experimental data collected for double microstructures containing PGPR-stabilised primary emulsions seems to corroborate the hypothesis in terms of experimental error. The role of PGPR in assisting/facilitating water migration<sup>(33)</sup> as well as the much smaller size of the PGPR-stabilised  $W_1$  droplets (Figure 1), would explain why the extent of this inaccuracy is greatly reduced for systems containing much larger primary droplets stabilised by the practically immobile EC particles (Table 1). Therefore, it is expected that even though these experimental inaccuracies would also affect the precision of the  $\phi''_{W_1}$  data for double microstructures containing EC-stabilised primary emulsions, their impact would be less pronounced. Regardless of its potential deficiency in accurately estimating the  $W_1$  content of the double emulsions studied here, DSC experimentation is still a valuable tool in terms of evaluating the impact of Pickering intervention on the microstructure formation and stability of  $W_1/O/W_2$  emulsions.



## CONCLUSIONS

The aim of this work was to assess the impact of Pickering intervention on  $W_1/O/W_2$  emulsions relevant to foods, by studying conventional surfactant-stabilised, partially Pickering-stabilised and solely Pickering-stabilised double architectures, produced under the same processing conditions. It was observed that double emulsion stability was extended via the employment of particles at either interface in comparison to the surfactant-only stabilised double emulsions. However, the compatibility/incompatibility between the emulsifiers/particles used is vital in double emulsion stability. It was found that if the two species employed were too similar in terms of their hydrophilic/lipophilic characteristics, double emulsion formation cannot be realised or becomes extremely unstable. The excellent stability of the EC/SSL double emulsions was proposed to, at least in part, relate to the Pickering functionality previously exhibited by SSL.<sup>(37),(40)</sup>  $W_1/O/W_2$  stability was even further enhanced by the adoption of a complete Pickering approach. Droplet size, light and confocal microscopy and DSC analyses, all confirm that EC/RH double emulsions retain a practically unchanged oil globule size and inner water content even one month after their formation. The results of this study also highlight the limitations of the DSC technique for analysis of the internal aqueous content of double emulsions in the absence of osmotic equilibration, as there was evidence of droplet migration during the calorimetry measurement. No attempts were made to osmotically balance the systems investigated here (at the point of formulating/forming these) and consequently future studies should focus on assessing whether the Pickering stabilisation advantages shown in the present study, persist when an osmotic opposition to the Laplace differential is introduced; thus, investigating Pickering stabilised double emulsions under both hypo- or hyper-osmotic conditions.<sup>(44),(52)</sup>

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## **ABBREVIATIONS**

CM, confocal microscopy; CMCC, colloidal microcrystalline cellulose; DE, double emulsion; DSC, differential scanning calorimetry; EC, ethylcellulose; HPMC, (hydroxypropyl)methyl cellulose; LM, light microscopy; LS, static multi-angle light scattering; NaCas, sodium caseinate; PE, primary emulsion; PGPR, polyglycerol polyricinoleate); RH, rutin hydrate; RSM, rotor-stator mixer; SD, standard deviation; SSL, sodium stearyl lactylate); T20, Tween 20 (sorbitan monolaurate).

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